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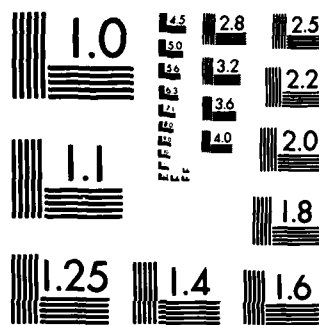
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INSTITUTE REPORT NO. 195

**BACTERICIDAL CAPABILITY AND RESPIRATORY BURST CHARACTERISTICS
OF THE MURINE MACROPHAGE CELL LINE RAW264.7**

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DIVISION OF RESEARCH SUPPORT

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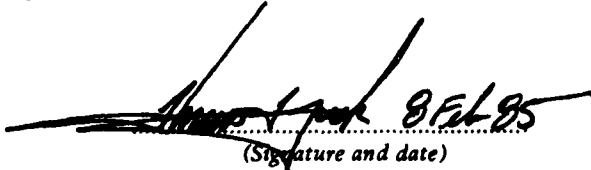
Bactericidal capability and respiratory burst characteristics of the murine macrophage cell line RAW264.7--McGown et al

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20. (Cont'd) ethylpiperazine-N-2-ethanesulfonic acid (HEPES) had only 15% of the TPA-stimulated oxygen consumption compared to cells cultured in the absence of HEPES. Time in culture affected the relative response of the cells to TPA and zymosan. Cells acquired from the American Type Culture Collection (ATCC) were at first more responsive to zymosan than to TPA, but after approximately 10 weeks in culture, the reverse was true. As the cells were carried in culture, extracellular release of superoxide and H_2O_2 changed with time and was independent of the magnitude of the respiratory burst. The differences in release of oxygen metabolites presumably reflected changes in the relative rates of generation, diffusion across the plasma membrane and intracellular disposition. The mechanisms by which culture conditions and history affected the magnitude of the respiratory burst are not known. Although the RAW264.7 cell line is potentially useful for a toxicity testing program, the sources of culture condition-dependent variability must be identified and controlled.

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ABSTRACT

The murine macrophage RAW264.7 cell line was used to establish assays of phagocyte functional status as part of an in vitro toxicity testing program. The variables included antimicrobial activity (toward *S. epidermidis*) and the respiratory burst (oxygen consumption, superoxide anion radical and hydrogen peroxide release) in response to 12-O-tetradecanoyl-13-phorbol acetate (TPA) and opsonized zymosan. The cells had potent antimicrobial activity. The magnitude of the respiratory burst in oxygen consumption was highly dependent on culture conditions. For example, cells cultured in a medium buffered with hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) had only 15% of the TPA-stimulated oxygen consumption compared to cells cultured in the absence of HEPES. Time in culture affected the relative response of the cells to TPA and zymosan. Cells acquired from the American Type Culture Collection (ATCC) were at first more responsive to zymosan than to TPA, but after approximately 10 weeks in culture, the reverse was true. As the cells were carried in culture, extracellular release of superoxide and H_2O_2 changed with time and was independent of the magnitude of the respiratory burst. The differences in release of oxygen metabolites presumably reflected changes in the relative rates of generation, diffusion across the plasma membrane and intracellular disposition. The mechanisms by which culture conditions and history affected the magnitude of the respiratory burst are not known. Although the RAW264.7 cell line is potentially useful for a toxicity testing program, the sources of culture condition-dependent variability must be identified and controlled.

Antimicrobial assays; respiratory burst; oxygen consumption; superoxide release; hydrogen peroxide release

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PREFACE

The work described in this report was initiated because we needed assays of phagocyte functional status to screen toxic effects of hazardous compounds. Because of the interest in respiratory toxicology, the original intent was to use pulmonary alveolar macrophages. However, experiments with alveolar macrophages were complicated by technical difficulties, e.g., impure and variable cell populations and unpredictable behavior depending on the history of the donor animal. We decided to use an established macrophage cell line to set up the assays and to train personnel. We chose the murine RAW264.7 cell line because it is easily maintained, grows rapidly, and literature reports existed which partially characterized its phagocytic function and respiratory burst. A technician (MGR) in the Analytical Chemistry Services Group maintained the cells for the first experiments. They were then transferred to the tissue culture facility within the Pathology Services Group where the culture medium and conditions were changed. This report summarizes our experiences with the RAW264.7 cell line.

ACKNOWLEDGMENTS

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Bactericidal Capability and Respiratory Burst Characteristics of the Murine Macrophage Cell Line RAW264.7 --McGown et al

Phagocytic cells (e.g. neutrophils, eosinophils and mononuclear phagocytes) are critical to the host's defense against invading pathogens. They accomplish their task by internalizing targets into phagocytic vacuoles and by subjecting them to various chemical oxidants and degradative enzymes. Among the events accompanying phagocytosis, is a dramatic increase in oxygen consumption and glucose oxidation (1,2). This "respiratory burst" does not provide energy for phagocytosis, but instead produces oxidants involved in microbial killing. An early event in the respiratory burst is the activation of an enzyme system that is dormant in resting cells. This enzyme system catalyzes the one-electron reduction of oxygen to form O_2^- , much of which reacts with itself, dismuting to produce oxygen and H_2O_2 . These species can then play a role in the generation of more reactive oxidants, including the hydroxyl radical and halide oxidation products (reviewed in references 1-5). The respiratory burst is an active area of scientific study, but the mechanism of triggering is not yet understood. A wide variety of physiological and pharmacological agents activate the oxidase, including F^- , detergents, chemotactic peptides and calcium ionophores. A particularly potent activator is the pharmacological agent present in croton oil, 12-O-tetradecanoyl-13-phorbol acetate (TPA). Activation is energy-dependent and reversible, does not require phagocytosis or degranulation, is preceded by a change in transmembrane potential, and requires calcium under some circumstances.

We used the macrophage cell line RAW264.7 to establish assays of phagocyte functional status. The variables we studied included bactericidal and phagocytosis capabilities, oxygen consumption, and O_2^- and H_2O_2 release. The bactericidal and phagocytic assays presented no major problems. However, measurements of respiratory burst yielded unexpected results. The respiratory burst characteristics not only depended on the culture medium, but also changed as the cell line was carried from generation to generation. These observations are summarized briefly in the following report.

METHODS

Sources of Cells

The RAW264.7 murine macrophage cell line was obtained originally through the courtesy of Dean Hefeman, PhD at Stanford University. A second sample of the cell line was purchased from the American Type Culture Collection (ATCC) (Rockville, MD).

Cell Culture Conditions

The cells were grown in closed flasks (T-75, Corning Glass Works, Corning, NY) at 37°C in Eagle's Minimum Essential Medium (MEM) in Earle's balanced salt solution supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 ug/ml gentamicin. Subcultures were prepared weekly by scraping with a rubber policeman and were seeded at a 1:4 split ratio into T-75 flasks. Cultures were refed every other day. The cells were harvested by replacing the medium with cell buffer from which Ca⁺⁺ and Mg⁺⁺ were omitted and to which 6 mM EDTA was added. Cell buffer consisted of 2.0 mM CaCl₂, 1.5 mM MgCl₂, 5.4 mM KCl, 1 mM Na₂HPO₄, 5.6 mM glucose, 120 mM NaCl, 0.2% bovine serum albumin (Pierce, Rockford, IL) and 25 mM hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES). During gentle shaking for 30 min at 37°C, most of the cells were released from the surface of the flask. The suspension was centrifuged at 200 X g for 10 min, washed once with, and resuspended in, the cell buffer. The cells (80-95% viable by trypan blue exclusion) were diluted to 6 x 10⁶/ml and maintained on ice until use.

When cells were grown routinely in MEM, a small amount (5 to 10 ml) of carbon dioxide was introduced into the culture flask at the time of subcultivation just before the flasks were tightly capped and placed in the incubator (i.e. closed flask system). This common laboratory technique prevented any alkaline spike in subculture pH. For some experiments, we used MEM supplemented by the manufacturer (Gibco, Santa Clara, CA) with 25 mM HEPES buffer for which carbon dioxide supplementation was unnecessary. (The sodium chloride content of the HEPES medium had been adjusted by the manufacturer to maintain an osmolarity of 290 milliosmoles.)

The closed flask system was used for all routine subcultivations. For certain experiments, cultures were maintained in loosely-capped T-75 flasks which allowed equilibration of the medium with a humidified 5% CO₂ - 95% air atmosphere (i.e. open flask system). Open flasks had less pH fluctuation than closed flasks and also a higher pH at culture confluence.

Bactericidal Assays

Bactericidal potency toward Staphylococcus epidermidis was assayed by the method of Mandell (6). For these assays, the cells were grown in RPMI 1640 medium (Gibco), with 10 mM (HEPES) buffer, 10% fetal calf serum and 40 ug/ml gentamicin sulfate. The cells were adherent to polystyrene culture flasks and were harvested by incubation for approximately 30 min with Earle's balanced salt solution without Mg^{++} or Ca^{++} (Gibco) containing 2 mM EDTA, followed by vigorous shaking. Cells were centrifuged at $200 \times g$ for 10 min and resuspended in cell buffer.

Respiratory Burst Measurements

Oxygen consumption was measured with a Gilson 5/6H Oxygraph and a Clark electrode. The system was calibrated by the method of Robinson and Cooper (7). To obtain stable baselines, the cells were prewarmed for 10 to 15 min before being diluted into the chamber to a final concentration of 3×10^6 /ml. Potassium cyanide (KCN) (1 mM) was included to eliminate mitochondrial respiration. The respiratory burst was triggered with either 0.05 uM TPA (Chemical Dynamics Corp., South Plainfield, NJ) or opsonized zymosan (Sigma; 3.3 mg/ml or approx. 1 mg/ 10^6 cells). Zymosan (25 mg/ml) was opsonized by incubation in serum (rabbit, porcine, or human) 30 min at 37°C, followed by centrifugation and resuspension to the same concentration in cell buffer. (For our purposes the source of serum was not critical and it was usually prepared in a batch and stored frozen in aliquots until use.) To measure H_2O_2 release, the cells were stimulated in the presence of horseradish peroxidase and scopoletin and the H_2O_2 -dependent oxidation monitored fluorometrically (8). O_2^- anion release was determined by measuring superoxide dismutase-inhibitable reduction of cytochrome c (9).

RESULTS

Bactericidal Potency (Stanford cells)

RAW264.7 macrophages had excellent bactericidal activity toward Staphylococcus epidermidis (Figure 1). The macrophages killed approximately 80% of the organisms within one hour and more than 98% by two hours.

Respiratory Burst (Stanford cells)

RAW264.7 macrophages responded to TPA with a vigorous increase in oxygen consumption (Figure 2). The assays were done in the presence of KCN to block oxygen uptake due to mitochondrial respiration. The inhibitor completely blocked basal respiration. TPA-stimulated oxygen

consumption was linear for 4 to 10 minutes and gradually slowed thereafter. The mean stimulated oxygen consumption in the presence of 1mM KCN was 3.4 ± 0.5 nmol/ 10^6 cells/min (Table 1).

These cells did not release O_2^- . This was not due to a faulty assay because we easily measured O_2^- release from other phagocytic cells (neutrophils). In contrast, we did detect H_2O_2 release, but only after a lag period of 1 to 4 min (mean = 2.6 min). After this lag period, H_2O_2 release occurred abruptly, as indicated by a sudden and dramatic decrease in scopoletin fluorescence.

The Stanford cells responded poorly to opsonized zymosan (Table 1). Oxygen consumption was only minimally stimulated by zymosan and there was no O_2^- or H_2O_2 release (Table 1).

ATCC Cells

Because the Stanford cells did not have a well-documented history (e.g. generation number), we obtained a new culture from the ATCC. Figure 3 illustrates the zymosan- and TPA-stimulated oxygen consumption in these cells as a function of time in culture. Zymosan-stimulated oxygen consumption was high the first week, but declined thereafter and remained between 1.5 and 2.5 nmol/ 10^6 cells/min for the remainder of the study. In contrast, TPA-stimulated activity began low (except for the first day) and increased thereafter. For the first 10 weeks, the cells consistently exhibited a greater respiratory burst in response to zymosan than to TPA, whereas for the remaining weeks, TPA-stimulated activity was greater.

During the first 10 weeks the ATCC cells did not release hydrogen peroxide in response to either TPA or zymosan. (O_2^- assays were not done, because it had never been detected previously.) After 10 weeks, the cells released both O_2^- and H_2O_2 in response to either stimulus. Thus the appearance of H_2O_2 and possibly O_2^- occurred at about the time when the cells became more responsive to TPA than to zymosan. The absolute amounts of O_2^- released did not reflect the amount of oxygen consumed because cells stimulated with zymosan released more O_2^- , whereas TPA-stimulated cells consumed more oxygen. Low H_2O_2 release was associated with long lag times (Table 1).

Effects of Culture Conditions

Culture medium affected respiratory burst parameters. Shortly after the Stanford cell line was transferred to the Pathology Services tissue culture facility, TPA-stimulated oxygen declined drastically. A systematic search for the cause revealed that the MEM culture medium containing HEPES buffer was responsible. Cells cultured in HEPES-containing MEM medium had approximately 15% of the TPA-stimulated oxygen consumption compared to cells cultured in the absence of HEPES (Table 2). The phenomenon was reversed within several days by

transferring the cells back into control medium. Several months later, the ATCC cells were inadvertently cultured in HEPES-containing medium for 5 days at which time their TPA-stimulated respiratory burst declined (Table 2, bottom line). HEPES had no effect if added to the medium on the day of the experiment.

ATCC cells grown in closed flasks (MEM medium) tended to have higher TPA-stimulated oxygen consumption than cells grown in open flasks, i.e. with the caps loose to allow equilibration of the gas phase with the external atmosphere (Table 3). We did not pursue the phenomenon, but it was possibly pH-related. At the time of cell harvest, the pH of the medium in closed flasks (6.6 to 6.75) was lower than in open flasks (7.24 to 7.4).

DISCUSSION

We found that RAW264.7 macrophages had excellent bactericidal capability. However, features of their respiratory burst showed large changes with variations with culture conditions and with time in culture. We have been able to identify some, but not all of the causes of the week-to-week variations in stimulated-oxygen consumption. The presence of HEPES in the MEM culture medium adversely affected oxygen consumption by the cells in response to TPA. Culturing the cells in closed flasks, which resulted in a decreased media pH, seemed to enhance TPA-stimulated oxygen consumption. Cell density and/or growth rate may have influenced these variables. When the ATCC cell line was begun, the cells grew slowly and the yield was low. These early experiments with the ATCC cells produced the highest zymosan-stimulated oxygen consumption we ever observed. The mechanisms for the above effects we observed are not known. It is tempting to speculate that less than optimal culture conditions (e.g. low pH) which cause slow growth rate produce cells with a greater respiratory burst. Cells grown in HEPES-buffered MEM looked more vigorous and healthy than their non-HEPES counterparts (subjective opinion of tissue culturist, BDS), but had much lower TPA- and zymosan-stimulated oxygen consumption.

TPA is a soluble pharmacological activator. Zymosan is a particulate cell wall preparation from S. cerevesiae and requires opsonization for activity. Stimulation with zymosan rather than TPA would seem to be more analogous to the situation in vivo where the macrophage encounters bacteria in the presence of humoral immune factors. During a period of approximately three months, when the RAW264.7 cells were passaged in culture, their response to zymosan decreased and the response to TPA increased. This phenomenon is not without precedence. The Stanford group found that their cells periodically grew less responsive to stimulation with IgG bound to haptenated liposomes. When this happened, they simply discarded the cells and thawed a fresh sample (D. Hafeman, PhD, personal communication).

Culture conditions and time in culture affected not only the magnitude of the respiratory burst, but also the amounts of O_2^- and H_2O_2 released into the extracellular medium. The cells obtained from Stanford released H_2O_2 , but not O_2^- . The ATCC cells initially did not release H_2O_2 or (we presume) O_2^- . After approximately 10 weeks in culture, the cells released both metabolites in response to either the particulate or the soluble stimulating agent.

The production of O_2^- by phagocytes, including macrophages, is well-documented. The amounts of O_2^- released vary widely according to cell type, species, physiological state and stimulus (1-5, 10, 11). Alveolar macrophages from several species release little or no O_2^- or H_2O_2 in response to phagocytosis (12). Compared to elicited or immunologically activated peritoneal macrophages, resident macrophages from the same anatomical site show relatively little activation of the respiratory burst or release of active oxygen metabolites following stimulation with a variety of agonists (5, 11, 12). Mouse resident peritoneal macrophages can be 'primed' in vitro by overnight incubation in appropriate medium. This treatment causes the cells to release seven times as much O_2^- in response to TPA compared to non-incubated cells (11). The amount of reactive oxygen intermediates released generally correlates closely with antimicrobial potency (5). This is also true of at least one macrophage cell line: A variant clone that released no O_2^- or H_2O_2 also had no antimicrobial activity (13). The RAW264.7 cells were unusual in that during the period of time when they did not release O_2^- , they did exhibit a strong burst in oxygen consumption in response to TPA as well as potent antimicrobial activity. Hafeman also detected no O_2^- release from RAW264.7 cells (personal communication).

Only small fractions of O_2^- and H_2O_2 generated by macrophages during the respiratory burst are released extracellularly (11). Ostensibly, this is due to intracellular metabolism of these species mainly by superoxide dismutase, glutathione peroxidase, and catalase. Thus, when we detected no release, it was probably because of complete intracellular degradation before detectable quantities diffused across the plasma membrane into the external milieu. Rossi and coworkers (14) measured several enzymes involved in formation and degradation of O_2^- and H_2O_2 in normal and Bacille Calmette-Guerin (BCG)-activated alveolar macrophages. (Macrophages from rabbits infected with BCG had enhanced respiratory burst and antimicrobial activity.) They found marked differences in the activity patterns. BCG activation increased the rate of O_2^- and H_2O_2 production more than the rates of degradation. The result was that, compared to nonactivated cells, the metabolites were released extracellularly in larger amounts, both as absolute values and as percentages of the amounts generated.

In the present study, culture conditions affected the respiratory burst of macrophages via unknown mechanisms. Presumably changes in intracellular disposition are responsible for some of the differences

in release of O_2^- and H_2O_2 . Some of the responses of RAW264.7 macrophages to TPA and zymosan were similar to peritoneal or alveolar macrophages. We report here, for the first time, that culture conditions dramatically affect the response of the RAW264.7 established cell line to these stimuli.

CONCLUSIONS

The established macrophage cell line RAW264.7 is easily maintained, grows rapidly, has potent antimicrobial activity and a vigorous respiratory burst in response to stimulation with TPA or zymosan. The cells are potentially useful for screening effects of hazardous compounds on phagocytic cells. Before this potential can be realized in a toxicity testing program, the sources of culture condition-dependent variability must be identified and controlled.

RECOMMENDATIONS

None.

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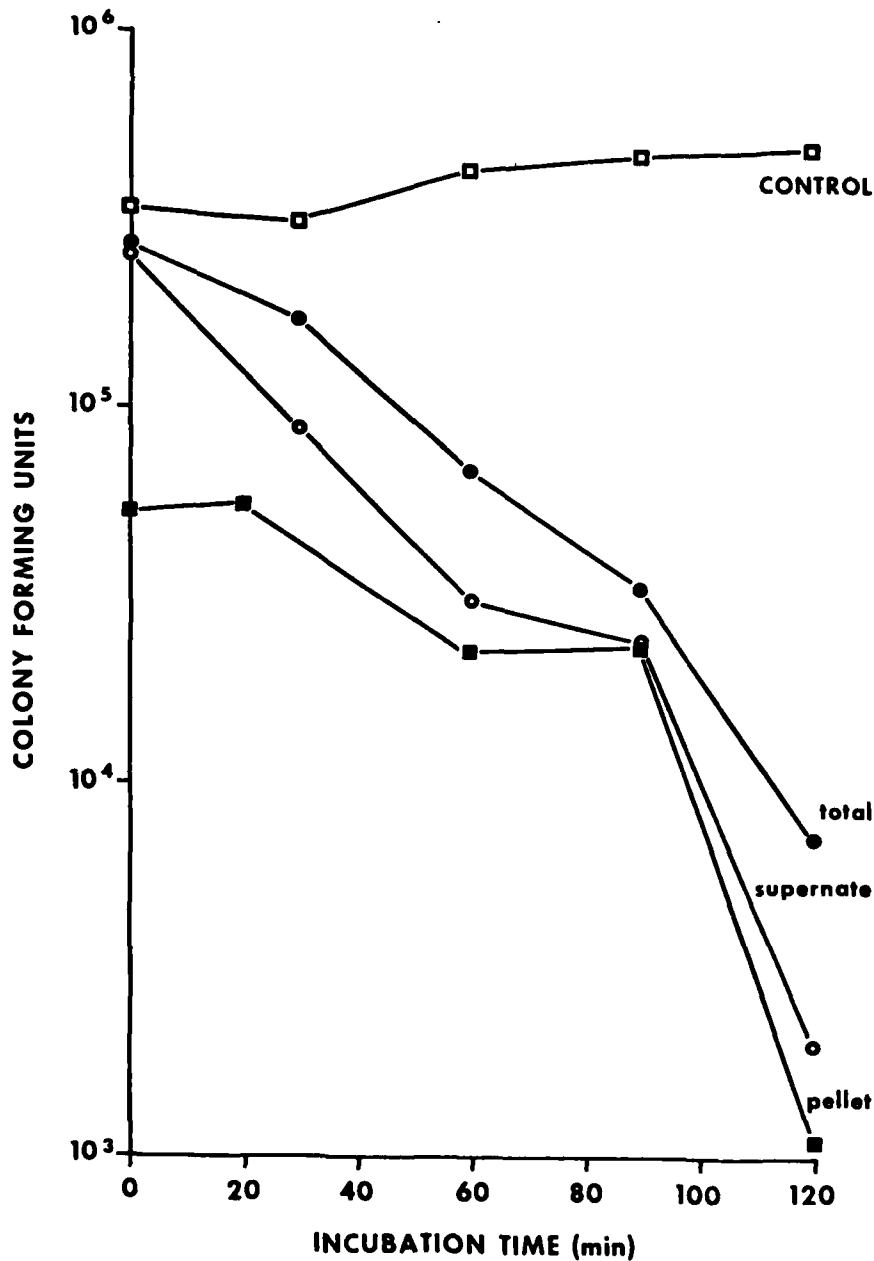


Figure 1. Bactericidal capability of RAW264.7 macrophages toward *S. epidermidis*. —□— control (no macrophages), —●— total (macrophages + bacteria), —○— bacteria not associated with macrophages (supernate after centrifugation), —■— viable bacteria associated with macrophages (pellet after centrifugation).

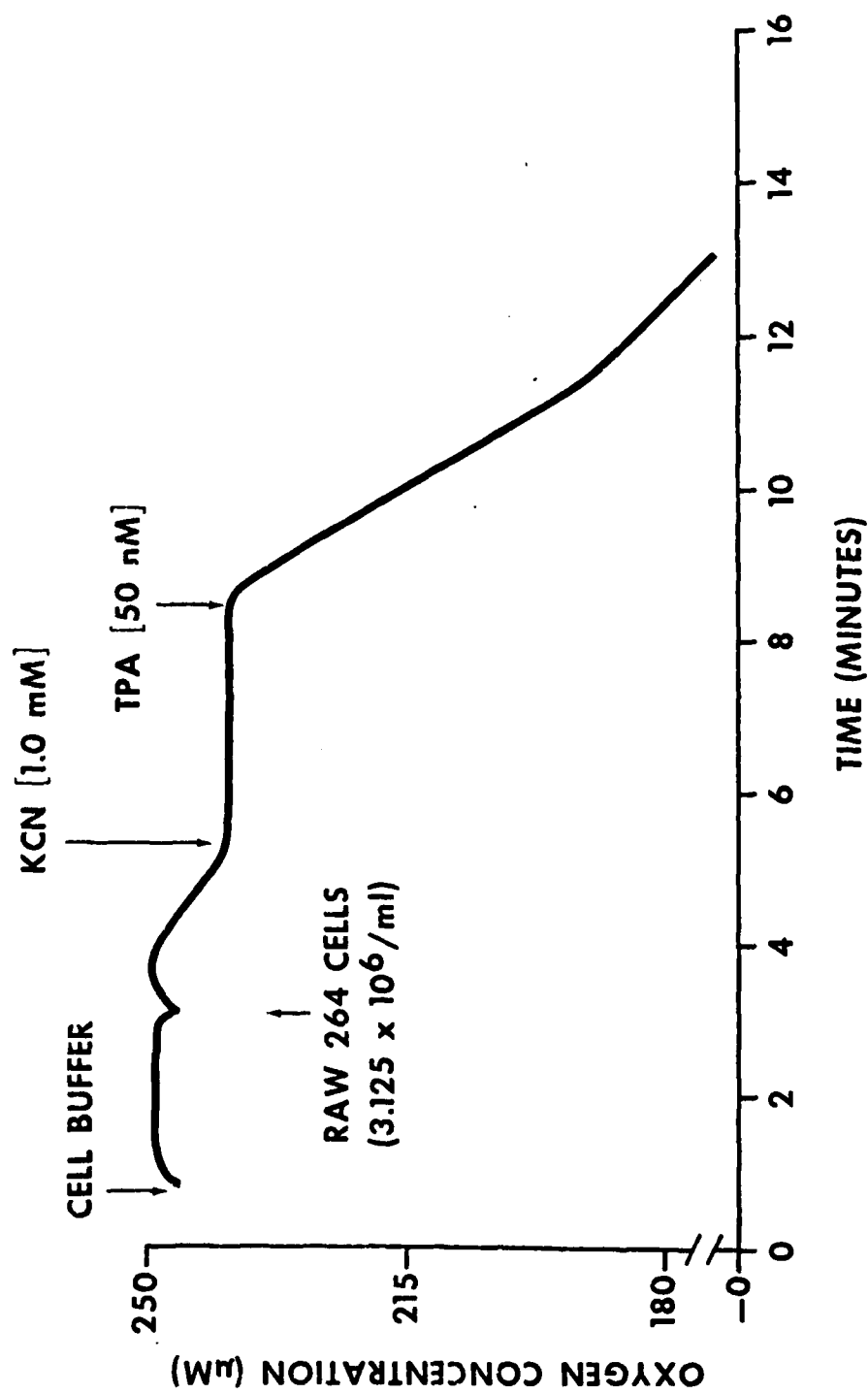


Figure 2. TPA-stimulated oxygen consumption by RAW264.7 macrophages.

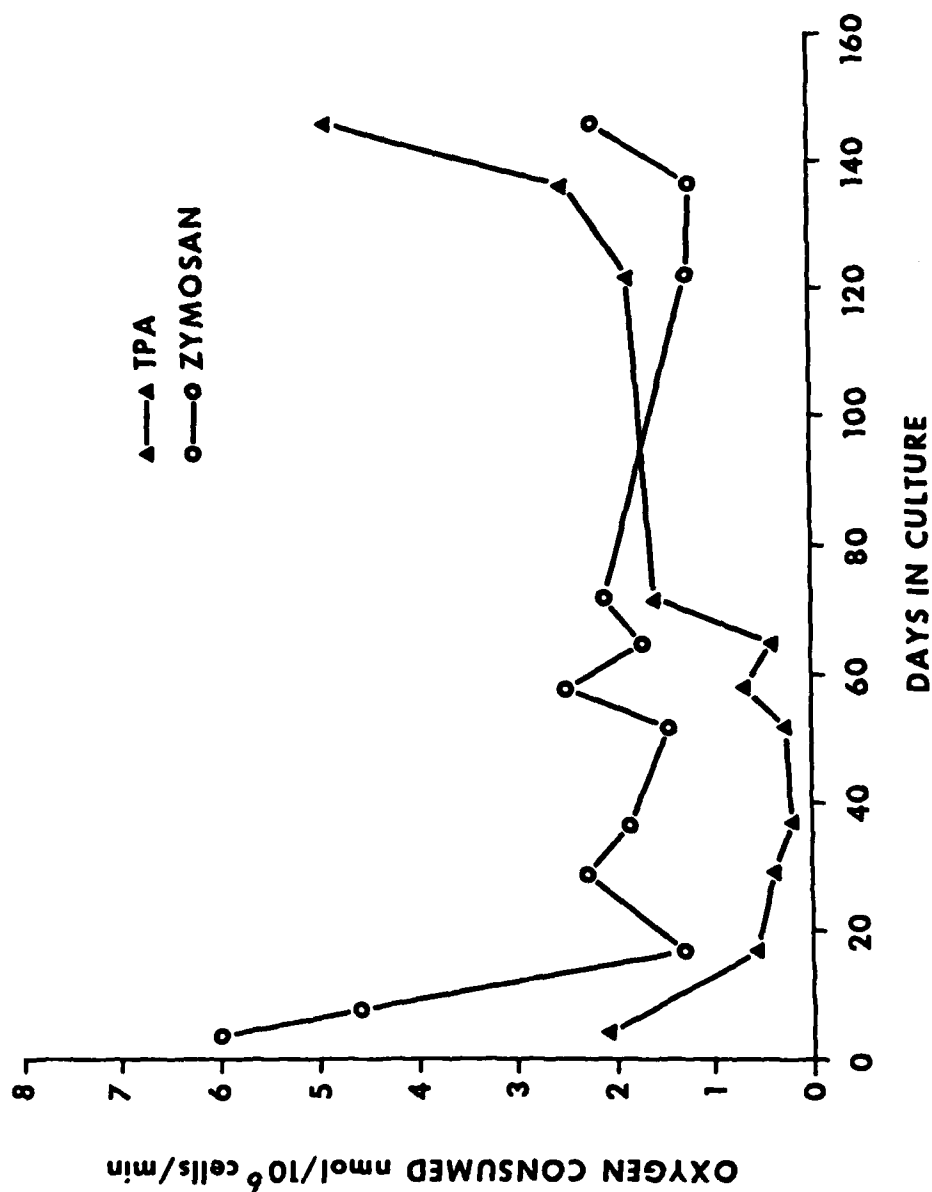


Figure 3. Oxygen consumption by RAW264.7 macrophages as a function of days in culture.

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Table 1. Oxygen consumption, superoxide release, and hydrogen peroxide release from RAW264.7 macrophages

Cells	Stimulator	Oxygen Consumption*	Superoxide*	Hydrogen Peroxide*	Lag
Stanford	TPA n=6	3.4 \pm 0.5	< 0.02	0.114 \pm .06	2-4 min
	Zymosan n=2	0.43	< 0.02	< .01	(> 8 min)
ATTC 1st 10 wks	TPA	0.66 \pm .61 n=7		< .01 n=3	
	Zymosan	2.71 \pm 1.60 n=8		< .01 n=5	> 15 min
ATTC 11-21 wks	TPA	2.71 \pm 1.30 n=4	.08 \pm .04 n=7	.14 \pm .09 n=4	3-7 min
	Zymosan	1.69 \pm 0.46 n=4	0.27 \pm .12 n=8	.08 \pm .07 n=4	13-17 min

*nmoles/ 10^6 cells/min, mean \pm S.D.

Table 2. Effect of HEPES in MEM culture medium on TPA-stimulated oxygen consumption by RAW264.7 macrophages

<u>Culture Conditions</u>		<u>Oxygen Consumption</u>
		nmol/10 ⁶ cells/min
		Mean + S.D.
- Hepes (n=12)		3.68 + 1.31
+ Hepes (n=8)		0.50 + 0.38
+ HEPES	- HEPES	
4 days culture (n=4)		1.44 + 0.37
7 days culture (n=4)		0.94 + 0.27
12 days culture (n=3)		4.79 + 1.41
- HEPES	+ HEPES	
5 days culture (n=3)		0.34 + .06

Table 3. TPA-stimulated oxygen consumption by RAW264.7 macrophages cultured in open and closed flasks

	<u>Oxygen Consumption</u>		<u>pH</u>	
	nmol/10 ⁶ cells/min			
	<u>Open</u>	<u>Closed</u>	<u>Open</u>	<u>Closed</u>
Expt 1	1.6	3.3	7.4	6.6
Expt 2	1.9	3.6	7.24	6.7
Expt 3	1.6	1.85	7.4	6.75

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